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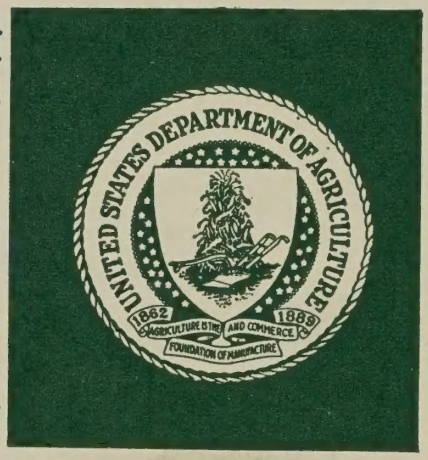

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PROGRESS REPORT

ARS AFLATOXIN RESEARCH ON PEANUTS

presented at

ARS-PEANUT INDUSTRY MEETING
May 2, 1972

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AGENDA

ARS-INDUSTRY MEETING ON PEANUT AFLATOXIN RESEARCH
May 2, 1972
Room 2096 South Agriculture Building, U. S. Department of Agriculture
Independence Ave., between 12th & 14th Sts., S. W., Washington

Chairman: F. R. Senti; Secretary: C. Golumbic

9:00 a.m.	Opening Remarks Developments Since Last Meeting	F. R. Senti, MNR
	Mycology of Peanut Molds	
9:10 a.m.	Southwest	H. W. Schroeder, MQ
9:25 a.m.	Southeast	R. J. Cole, MQ
9:40 a.m.	Discussion	
	Field Studies-Prevention on the Farm	
9:45 a.m.	Genetic Resistance-Natural Inhibitors	W. K. Bailey, PS
10:10 a.m.	Harvesting, Drying, Curing Practices	J. L. Butler, AE
10:30 a.m.	Discussion	
10:45 a.m.	Break	
	Storage Studies	
11:00 a.m.	Storage Studies	C. E. Holaday, MQ J. W. Dickens, MQ
11:20 a.m.	Discussion	
	Sampling	
12:00 noon	Update	T. B. Whitaker, MQ
12:20 p.m.	Discussion	
12:30 p.m.	LUNCH	
	Analytical Methodology	
1:30 p.m.	Millicolumn	C. E. Holaday, MQ
1:40 p.m.	Developments in Analytical Methodology at SMN	W. A. Pons, SMN
2:00 p.m.	Comments from AMS	J. B. Wegener, AMS
2:15 p.m.	Comments from FDA	A. D. Campbell, FDA
2:30 p.m.	Discussion	
	Removal and Inactivation	
2:40 p.m.	Removal, Inactivation, and Protective Factors	H. L. E. Vix, SMN
3:00 p.m.	Discussion	
3:15 p.m.	Break	
3:30 p.m.	Comments from Research Committee of the National Peanut Council	G. F. Hartnett, N
4:00 p.m.	Closing Remarks	F. R. Senti, MNR
4:15 p.m.	Adjourn	

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Mycology of Peanut Molds - Southwest

H. W. Schroeder

The problem of aflatoxin contamination in peanuts can and is being attacked under two broad action categories. They are (1) preventing contamination and (2) preventing contaminated peanuts or peanut products from reaching the consuming public. Of these two approaches, the second has been most successful but almost everyone will agree that the first category, prevention of contamination, is the most desirable. Improved harvesting and conditioning methods have and will continue to decrease the prevalence of severe aflatoxin contamination in peanuts but it seems probable that the problem will continue unless breakthroughs in research lead to new and different approaches to the problem.

It is my opinion that we have insufficient knowledge of the fungi primarily responsible for the production of aflatoxin, the Aspergillus flavus group. This group not only includes several distinct species but within the two species that regularly produce significant quantities of aflatoxin, we have a diverse series of strains that vary widely in their ability to produce aflatoxins and in their relation with peanuts and other crops. In our laboratory, we have been studying these fungi with the primary goal of determining the relations between the diverse strains and particular crops in our area. Although we here are most interested in peanut, the data I am presenting compares our data from peanut with that of three other crops: Cottonseed, Rice, and Sorghum. These studies are in the third year but our results the past two years have been essentially the same as those of the 1969-70 crop year. Peanut samples were collected at the shellers and all grades including pickouts were included in the study. Our purpose was to study the fungi and every effort was made to obtain low grade samples of all four crops.

We found that peanuts are invaded more often by the A. flavus group than are the other crops. In our samples, these fungi were isolated from an average of 12.4% of the peanut kernels. The percentage of infection per sample ranged from 0 to 90%. Of the other crops, A. flavus was most prevalent in rice with an average of 2% infested kernels and prevalence ranging from 0 to 65%. (Table 1)

Aflatoxins were detected in 9 of 56 samples tested (ca 17%). The concentrations of the toxins ranged from < 2 to 57 ppb with an average per contaminated sample of 17 ppb and 3 ppb overall average. In contrast, aflatoxins were not detected in rice, the crop in which prevalence of A. flavus was next to peanut. (Table 2)

Randomly selected isolates of the A. flavus group were tested to determine their ability to produce aflatoxin. Of the isolates from peanut, 98% produced aflatoxin, compared to 20% of the rice isolates. Peanut isolates also tended to be more toxigenic with many isolates producing 3000 µg per flask while the most toxigenic from rice only produced 1100 µg. (Table 3)

In addition, peanuts were the better substrate of the four crops for aflatoxin production. Most isolates irregardless of their toxigenicity and of the source from which they were isolated, tended to produce more aflatoxin on peanut than on the other substrates. (Table 4)

When classified according to species, most isolates from peanut were A. flavus (97%), 2% were A. tamarii and 1% were A. parasiticus. (Table In the Southwest, we find that A. flavus produces only the B aflatoxins and their derivatives. A. parasiticus produces both the B and G toxins while A. tamarii has never been found capable of producing more than a trace of B_1. Our most toxigenic strains are in the A. flavus species, with some isolates producing very large amounts of aflatoxin B_1; although some A. parasiticus strains may produce more total toxins (B + G).

It is apparent from these data that of the major crops in the Southwest, peanut is most susceptible to contamination with aflatoxin. The prevalence of kernels infested with A. flavus is greatest in peanut and peanuts appear to be the best substrate for the production of the toxins. Another major factor is that isolates of the A. flavus group associated with peanut are generally toxigenic and have a greater average toxigenicity than those of the other crops. We therefore looked for a unique characteristic of peanuts that could have a bearing on this phenomenon.

The most obvious difference between peanut and the other crops is that peanut fruits develop in the soil, while the seed of the others are borne on aerial portions of the plants.

The A. flavus group is commonly found in soil. Information on its prevalence in the soil and its relation to peanut culture is quite limited. It is difficult to find broad principals that apply to such aspects of soil microbiology because of the great variability in soil types, soil composition, and in all the other factors that affect the soil environment. However, it seemed probable that the soil serves as a reservoir of A. flavus inoculum and thus has a primary effect on any future contamination with aflatoxin of crops grown in that soil.

As part of our cooperative effort with the Texas Agricultural Experiment Station, we have been working with Dr. R. E. Pettit, TAES on this

aspect of the problem. Starting in May of 1971, Dr. Pettit has been sampling soils at six sites and three locations. Samples are taken at depths of 1 to 2, 5 to 8, and 10 to 12 inches at each site and intervals of one month. Counts of the mycoflora of each sample are made by the dilution plate method and cultures of the A. flavus group are transferred for further study. Our part of this experiment is to test the cultures to determine their ability to produce aflatoxins.

Although the study has not been completed and the data has not been analyzed, several definite trends have appeared. Counts of A. flavus were low in all soils in May but increased during the summer, peaking in August and September. A. flavus increased more in soils planted to peanut than in soils planted to the cereals. These fungi remained almost completely absent in soils of one site that had been in grass for many years. Generally, A. flavus populations decreased as depth of soil increased.

Our studies of the cultures isolated from the soil showed that most cultures produced aflatoxins regardless of location, site or depth of soil. A partial summary of our results indicates that 96% of the isolates produce aflatoxins in detectable amounts in our test procedure and 34% can be considered as highly toxic, producing at least 1000 µg of B_1 per flask. The average amount of B_1 produced per culture exceeded 1000 µg per flask, indicating an overall average high toxigenicity of these cultures from the soil. By species the distribution is 78% A. flavus, 6% A. parasiticus, and 16% A. tamarii. Comparing these figures with the data obtained by testing isolates from peanut, we find them in very good agreement. Thus, it appears that the soil is the chief source of A. flavus inoculum for peanuts. Moreover, referring back to our study of A. flavus from rice, only 20% of the isolates were toxin-producers. Since rice is grown in flooded fields and combined when the soil is still wet, rice seldom comes in contact with soil particles, yet newly combined rice is usually well dusted with A. flavus spores. I suggest the most probable source of these spores is the dead plant parts of the rice. This suggests that the soil as a medium tends to favor the retention and propagation of toxigenic strains of the A. flavus group. The final point I wish to make is that prevention of aflatoxin contamination in peanut should start with controlling the inoculum level in peanut soils. Moreover, crop rotation appears to be one of the methods by which inoculum may be decreased.

TABLE 1

Prevalence of species of the *Aspergillus flavus* group isolated from seed of four field crops in 1969 - 70.

Crop	Samples	*Aspergillus flavus* group in seed	
		Avg/sample	Range in samples
	no.	%	%
Peanut	56	12.4	0 - 90
Cottonseed	73	0.7	0 - 7
Rice	134	2.0	0 - 65
Sorghum	122	0.4	0 - 5

TABLE 2

Aflatoxin in four field crops in 1969 - 70.

Crop	Samples tested	Aflatoxin			
		Positive samples	Range	Avg/contaminated sample	Avg/sample tested
	no.	no.	ppb	ppb	ppb
Peanut	56	9	<2 to 57	17	3
Cottonseed	73	9	<2 to 156	65	8
Rice	134	0	0	0	0
Sorghum	122	0	0	0	0

TABLE 3

The percentage of aflatoxin-producing isolates of the Aspergillus flavus group isolated from four field crops (1969 - 70).

Source	Isolates tested	Isolates producing aflatoxin	Maximum yield[1/] of aflatoxin B_1
	no.	%	µg/flask
Peanut	100	98	3300
Cottonseed	59	81	3200
Rice	127	20	1100
Sorghum	63	24	3300

1/ Yield is based on tests on four substrates consisting of 3g of ground peanut, cottonseed, rice, and sorghum, respectively, suspended in 50 ml of water in 250 ml Erlenmeyer flasks. Yields are rounded to two significant numbers. Incubation time 7 days; temperature 25° C.

TABLE 4

The aflatoxin producing ability of cultures of the Aspergillus flavus group isolated from four field crops in 1969 - 70.

Source	Aflatoxin B_1 produced on:[1/]			
	Peanut	Cottonseed	Rice	Sorghum
crop	µg/flask	µg/flask	µg/flask	µg/flask
Peanut	1100.0	210.0	570.0	370.0
Cottonseed	700.0	160.0	360.0	260.0
Rice	18.0	3.2	12.0	4.7
Sorghum	160.0	29.0	91.0	63.0

1/ Given as the average production/isolate tested. Yields are rounded to two significant numbers. Substrates consisted of 3g of the respective ground seed in 50 ml of water per flask. Incubation time 7 days; temperature 25° C.

TABLE 5

The relative prevalence of species of the Aspergillus flavus group in randomly selected isolates from four field crops (1969 - 70) tested for aflatoxin producing ability.

Source	Species of the A. flavus group			
	A. parasiticus	A. flavus	A. oryzae	A. tamarii
	%	%	%	%
Peanut	1	97	0	2
Cottonseed	4	92	0	4
Rice	0	100[1/]	0	0
Sorghum	0	100	0	0

1/ 21 isolates appeared to be intermediate forms between A. flavus and A. oryzae or mixtures of the two species, although the morphology most strongly corresponded to the description of A. flavus.

Mycology of Peanut Molds - Southeast

Richard J. Cole

A four phase research program at the National Peanut Research Laboratory designed to evaluate the presence or absence of new potential mycotoxin problems on peanuts has been in effect for the past two growing seasons. The program consists of (1) screening peanuts for actual and potential microbial contamination, (2) evaluating the mycotoxin producing potential of these isolates, (3) purifying the metabolites responsible for toxicity and (4) characterizing the metabolite(s) as to its chemical structure. The major objective of this program is to determine if any potentially new mycotoxin problems exist on peanuts and to thus enable an accurate evaluation of the presence or absence of these mycotoxins in food or feed supplies.

A mycotoxin producing mold, identified as Penicillium verruculosum, was detected on peanuts that were part of a test concerned with the effects of various relative humidities on stored peanuts. Extracts of the mold culture produced severe tremors and acute toxicity in one-day-old cockerels. The I.P. LD_{50} of the purified mycotoxin was 2.45 mg/Kg in mice and 15.20 mg/Kg in chicks. Orally administered LD_{50} values for the toxin were 126.7 mg/Kg in mice and 365.5 mg/kg in chicks. The trivial name "verruculogen" is proposed for this tremorgenic mycotoxin. Physical and chemical characteristics of the toxin have demonstrated that it differs from the other known tremorgenic compounds.

An extremely toxic isolate of Fusarium moniliforme Sheldon was found as part of a screening study by Dr. Ben Doupnik and Dr. John Peckham at Tifton. In co-operative studies we isolated the major toxic metabolite. The toxin was a crystalline water soluble compound with an LD_{50} of 4.0 mg/Kg in one-day-old cockerels dosed orally with an aqueous solution of the toxin. This is approximately the same level of magnitude as aflatoxin B_1 in our chick bioassay. In addition to vertebrate toxicity the metabolite demonstrated growth regulating and phytotoxic effects on higher plants. Foliar application at 2000 and 200 ppm on corn and tobacco plants produced necrosis, interveinal chlorosis, stunting and other morphological irregularities. At 200 and 20 ppm cucumber roots were inhibited 68 and 19% respectively. Wheat coleoptiles were inhibited 57% and 24% at 200 and 20 ppm. Chemical and biological data suggest a structurally new growth inhibitor with potential utility as a chemical pruning agent. These studies were performed in co-operation with Dr. H. G. Cutler, Research Plant Physiologist at the Coastal Plain Experiment Station in Tifton. Future co-operative field studies on effects of this metabolite on peanut, cotton and tobacco plants are anticipated.

Discussion:

I. An unidentified participant asked about the theory proposing that virus carrying strains are the only strains of the A. flavus group that produce aflatoxins. My reply was that the bulk of the evidence indicates a simpler genetic basis for aflatoxin production. The burden of proof lies with the proponents of the virus theory.

II. Walter Pons, SMN, raised the question of chemical soil treatment for control of A. flavus. Answer: Usually not economical or highly effective. Often introduces new problems. Is a possibility that cannot presently be ruled out.

III. Dr. Senti introduced discussion on grassland for peanut and grass-peanut rotation. Answer: Our present data indicates a possible beneficial effect. Observations of peanut farmers also tend to support this view.

Field Studies - Prevention on the Farm
Genetic Resistance-Natural Inhibitors

W. K. Bailey

Peanut pods and seed develop in the soil in a veritable microbiological jungle, and many of the members of this miniworld can invade plant tissue. In order to survive and to develop to maturity in the soil, peanut pods and seed must be endowed with a wide range of characteristics that enable them to resist invasion by the microorganisms, or to inhibit their development if invasion occurs. Apparently sound peanut seed inside apparently sound pods are invaded by numerous fungi and bacteria. Fortunately, under most circumstances these invaders remain quiescent and can be described as nonpathogenic inhabitants. This fungal and bacterial invasion begins early in the life of the developing fruit. The microbial population is a dynamic one, changing in both components and their prevalence as fruit development proceeds.

Frequently Aspergillus flavus is among these nonpathogenic invaders of peanut fruits. A. flavus has been reported in elongating pegs before they reach the soil. Early invasion of developing pegs and peanut fruit by A. flavus is not necessarily a cause for alarm. Regardless of when invasion occurs, A. flavus usually remains quiescent during the development and maturation of the pods and seed, and subsequently during curing provided seed moisture is reduced at a steady rate to a level that is safe for bulk storage.

Natural inhibitors doubtless are responsible for the failure of A. flavus to proliferate and produce aflatoxins during fruit development and maturation, and during curing under favorable conditions. These natural inhibitors might be an attribute(s) of the peanut fruit tissue, or their origin might be one or more of the other microorganisms that inhabit the fruit either by production of metabolites or by competition for nutrients.

I shall review briefly some of the highlights of research that has been undertaken to provide us with a better understanding of the nature of these assumed natural inhibitors of A. flavus in peanut fruits, and to determine whether certain genotypes might be more effective than others in inhibiting invasion of pods and seed by the fungus or in insuring that the fungus remains quiescent if invasion occurs. My discussion will involve results of research by our own staff in cooperation with Auburn and Oklahoma State Universities, and research carried out under grants or cooperative agreements with Texas A & M, Colorado State, New Mexico State, and North Carolina State Universities, Virginia Polytechnic Institute and State University, and the University of Florida.

In Colorado peanuts were grown under nearly germ-free conditions and the developing fruits were inoculated with A. flavus and a number of other fungi singly and in various combinations. The pods and seedcoats were successfully inoculated with A. flavus, but the embryo proper resisted invasion by the fungus. Naked embryos inhibited the development of A. flavus when grown on heavily inoculated agar media. Inhibition of another fungus, Trichoderma viride, under similar circumstances was even more striking than for A. flavus.

In subsequent research at Las Cruces, New Mexico, the existence of this inhibition of A. flavus by fresh naked embryos was confirmed. Four inhibitors of A. flavus have been detected in the acetone extract of cotyledons of freshly harvested peanuts. Three of the chemicals appear to be acidic phenolic-type compounds, and the 4th appears nonacidic. Repeated efforts to detect such inhibitors in dried seed were unsuccessful. A. flavus readily colonized the intact surface of dried seed and seedcoats thereof, but did not colonize uninjured cotyledon tissue of 3 botanical varieties of peanuts when growing on an agar medium. Broken pieces of cotyledons of dried seed were heavily colonized by A. flavus.

Perhaps the presence of these inhibitors in fresh embryos might be a partial explanation for the findings of several investigators that aflatoxins are rarely found in fresh peanut seed that are high in moisture regardless of drying conditions. And the apparent absence of such inhibitors in dried seed might explain, in part, the increased vulnerability of peanut seed of about 30% and lower moisture, to contamination by aflatoxins under adverse drying conditions.

In Texas a high proportion of peanut seed was found to contain Bacillus subtilis growing both within and between cells in subepidermal cotyledonary tissue. Efforts to grow germ-free peanuts in Colorado were successful until plants were about 50 days old, at which time B. subtilis appeared from an as yet unexplained source. There is some evidence that B. subtilis might be systemic in peanut plants. This bacterium is a known antibiotic producer and has been reported to act as an antifungal agent. B. subtilis was found to reduce pod and seed invasion by A. flavus when plants were grown under gnotobiotic conditions. B. subtilis also reduced A. flavus colonization of flower parts, which might be a source of the fungus that invades pegs before they enter the soil. Our financial support of this research in Colorado is scheduled to end next month because of lack of funds, with many intriguing questions still unanswered.

Although seed of present varieties of peanuts have characteristics that inhibit invasion by A. flavus and inhibit proliferation of the fungus when invasion does occur, all of our commercial varieties under certain circumstances are highly vulnerable to contamination by aflatoxins. An urgent need exists for the development of desirable commercial varieties that are more resistant to A. flavus than varieties

now grown. Research towards such an end began in 1968 in cooperation with the Alabama Agricultural Experiment Station, with funds made available by the Department of Health Education and Welfare. Results have been encouraging, but not yet conclusive. We have found that recently-cured hand-shelled dry seed of certain peanut genotypes resisted invasion by A. flavus when the seed were reimbibed to about 35% moisture, heavily inoculated with spores of the fungus, and incubated at 25°C for 7 days in petri dishes under circumstances where they gradually lost moisture. Seed of other genotypes handled in a similar manner were heavily invaded by the fungus. Comparably handled seed of commercial varieties were intermediate in their response. Genotypes that we classify as resistant generally show from 0 to about 10% of seed invaded, in sharp contrast to the highly susceptible genotypes with 80 to 100% invasion, and standard commercial varieties ranging from about 40 to 60%.

These differences among genotypes in seed invasion have been found to be highly reproducible when the plants are harvested near optimum maturity. Resistance tends to break down when digging is delayed several weeks beyond optimum maturity. The two most resistant accessions, Valencia type peanuts from Argentina, have shown a consistent low level of seed invasion through 4 generations, with little evidence of segregation for resistance among individual plant selections. Another accession, a Virginia type from India, has shown moderate resistance through 4 generations.

The nature of this resistance has not been determined. However, circumstantial evidence indicates that intact seedcoats probably are involved. When the seedcoat of seed that resisted invasion was pricked with a needle, A. flavus developed rapidly in the area of the injury.

Results from a similar screening program begun in Florida in 1970 confirm the Alabama results. Several Florida breeding lines have shown significantly lower levels of seed invasion by A. flavus than seed of commercial varieties. Florida plant pathologists have postulated that an intact seedcoat is required for this tolerance and appears to function as a mechanical barrier to penetration by the fungus.

Results of some unrelated research in Oklahoma might have implications in relation to the nature of this resistance or tolerance of seed of certain genotypes to invasion by A. flavus. Young developing embryos of peanuts are surrounded and nourished by endosperm that is richly endowed with potent growth promoting substances. Until recently mature seed of peanuts were considered to have no endosperm, or at most just a few crushed remnants of cells between the seedcoat and the cotyledons. Recent research at Oklahoma State University indicates that at maturity the surface of peanut cotyledons is covered completely by a single-cell layer of endosperm. If this is confirmed, we should

consider the possibility that in the pricking or abrading of seedcoats in Alabama and Florida, cells of the cotyledons might have been injured sufficiently to give the fungus access to tissue below both the seedcoat and the endosperm.

We need to identify the nature of this resistance so that we can know what we are working with and what to look for in future screening to identify a greater degree of resistance than that now available. Towards such an end we are negotiating with Texas A & M University for cooperative research on biochemical and structural components in resistant and susceptible peanut genotypes as related to seed invasion by Aspergillus flavus. This research is in cooperation with the Departments of Plant Sciences and Biochemistry and Biophysics, and will involve a research team consisting of a plant physiologist, a biochemist, and a plant pathologist. In this research an attempt will be made to relate biochemical, structural, and ultrastructure characteristics of seed of different genotypes at different stages of development, including fully mature seed, to extent of seed invasion by A. flavus.

Thirty accessions of wild Arachis were included in our screening operations in Alabama. In an initial test, seed invasion ranged from 0 to 100%. Five accessions showed invasion no higher than 10%, and 6 showed 100% invasion. Contrary to the experience with other crops, we have found little usable genetic resistance to pests in cultivated peanuts. Certain wild Arachis species have shown high resistance or immunity to several major diseases and to northern rootknot nematodes. The wild Arachis accessions that have these highly desirable attributes cannot be crossed successfully with cultivated peanuts.

Intensive cooperative research to remedy this situation is under way at Oklahoma State and North Carolina State Universities. A new grant is being negotiated with N. C. State under which we would continue for another 2 years our financial support for research on the development of pathways of gene flow from wild species of Arachis to cultivated peanuts. Success in this basic research could have important implications for our problem with aflatoxins in peanuts, if we find immunity to A. flavus in any of the estimated 300 accessions that are present in this country.

We are uncertain as to just how useful resistance to A. flavus of the sort detected in cultivated peanuts might be under conditions of commercial production of the crop. However, crosses have been made already between the most resistant and the most susceptible genotypes to determine how resistance is inherited. And crosses have been made between the most resistant lines and our most productive varieties and advanced breeding lines of the 3 major market types in an effort to develop desirable commercial varieties with tesistance to A. flavus.

Meanwhile extensive screening of new genotypes for resistance continues. Thus far some 500 genotypes out of an estimated 5,000 to 6,000 have been screened. The number of new peanuts that we are screening each

year probably does not exceed the new genotypes that are being developed each year by breeding in this country and abroad. Consequently we urgently need a more rapid procedure for screening for resistance.

In our search for resistance we are exploring to what extent the resistance detected in the dry seed might be operative in seed inside freshly harvested pods that are losing moisture under conditions that are conducive to proliferation of A. flavus. Results thus far indicate a relationship, but findings are not yet conclusive.

In Virginia where a study is being made of the role of pod exudates in colonization of fruits by A. flavus, conidia in soil adjacent to intact pods failed to germinate, but conidia in soil adjacent to injured pods germinated readily. In most of the work reported herein, resistance to A. flavus appears to be associated with physically intact tissue.

Unless some new type of resistance or immunity is found, it seems illogical to expect A. flavus resistant varieties to solve our aflatoxin problem completely. However, if use of resistant varieties could be combined with avoidance of fields that are aflatoxin prone, avoidance of cropping patterns that might predispose the crop to aflatoxin contamination, digging at optimum maturity, gently removing pods from plants at the time they are dug, and drying the pods in such a manner that all pods in a given lot will be losing moisture simultaneously, aflatoxin contamination of peanuts that reach the market would be reduced to a level well below that of the past few years. In fact, in my estimation, resistant varieties plus precision use of the above procedures with our entire crop could largely eliminate aflatoxins in peanuts that go to market.

-- Field Studies-Prevention on the Farm Harvesting, Drying, Curing Practices

J. L. Butler

Harvesting peanuts at optimum maturity may be one way of reducing the risk of aflatoxin contamination. Today, however, there is not a good objective measure of determining when to dig peanuts. Cooperative research with MQRD has been conducted for two years in this area. Two methods, which are fast and simple are being tested further. For one, the electrical impedance at 5 and 500,000 hz is measured and the ratio calculated. For the second method, the freshly harvested peanuts are ground in methanol, filtered and the light transmission at 450 mμ measured. Both of these methods show promise and hopefully one of them can be further developed to provide the farmer with the answer of "when is the optimum time to dig".

There has been some evidence to indicate that aflatoxin may, under certain conditions, be present at the time peanuts are dug. Cooperative studies with MQRD were set up to see whether conditions known to produce aflatoxin under laboratory conditions would produce it in the soil prior to digging. A section of the field was covered with a polyethylene tent and the following treatments applied: 1) normal conditions (no cover), 2) high soil moisture maintained by adding water for 4-weeks prior to digging (no cover), 3) drought conditions with no water applied for 4-weeks prior to digging, and 4) drought conditions from 4-weeks to 1-week prior to digging, then, high soil moisture maintained until digging. High rainfall maintained a soil moisture of 8-11% for treatments 1 and 2. Soil moisture for 3 and 4 steadily decreased until the end of 3-weeks when moisture was added to treatment 4 bringing the moisture up to about 9% where it was maintained until digging one week later. Although these conditions would be expected to produce aflatoxin, none of them did.

Propionic, propionic-acetic acid and dichlorovos were applied to inverted Spanish peanuts immediately after digging. Rates of 5- and 10-pounds per acre of the acid were applied and rates of 1, 2 and 4-pounds per acre of dichlorovos were applied in each of 2 liquid formulations and one granular formulation. Residue samples were taken

immediately and again at the end of 24 hours. Aflatoxin samples were taken at the end of 7 days. The results were enconclusive as no aflatoxin was found in any sample, including the check.

Propionic and propionic-acetic acid were applied to freshly harvested peanuts which were held for 5 days without drying. Under these conditions, they were ineffective in preventing aflatoxin. The various treatment levels, ranging from 1/8% to 1/2%, had aflatoxin ranging from 156 to 405 ppb.

Using CO_2 production as an index of the deterioration rate, the rate increased by 45% for each 10°F temperature change between 65 and 85°F. Peanuts innoculated with A. flavus (initial m.c. 55%) graded segregation III after 112 and 106 hours exposure to 64 and 80°F respectively.

Studies of the growth of A. flavus on peanut seed with varying degrees of hull damage show greater infection of seed in damaged pods. Seed from invisibly damaged pods were almost as susceptible as those from visibly damaged pods.

The development of a harvester for green peanuts is continuing both at Tifton and Holland. Such a harvester would eliminate the field exposure and its attendant aflatoxin risk. Although efficiencies as high as 96% were obtained at Holland, efficiencies ranging from 85% upward were the more common at Tifton. Damage in both mechanisms is comparable to that of hand picking. Additional work needs to be done on separating mechanisms for these.

For a green harvester to be practical, the dried product must be acceptable. Work at Tifton (cooperatively with MQRD) indicates that programming for 120°F exposure during part of the drying cycle may give better flavor development than the 95°F maximum now observed.

Discussion: (W. K. Bailey)

QUESTION: At one time you were pessimistic about the prospects for development of peanut varieties that are resistant to Aspergillus flavus. How do you feel now about prospects for such a development?

ANSWER: In 1968 when we initiated research to develop desirable commercial varieties of peanuts with resistance to Aspergillus flavus, I was pessimistic about success. However, we have detected apparent resistance to A. flavus in two Valencia type peanuts introduced from Argentina. This has changed my outlook to the extent that now I am confident that improved varieties of peanuts with resistance to A. flavus can be developed. Breeding work is underway already to incorporate the resistance in these Valencia peanuts into desirable commercial varieties of our 3 major market types of peanuts.

However, with the sort of resistance detected thus far, other procedures for minimizing aflatoxin development would need to be used along with resistant varieties if such varieties are to have a major impact on aflatoxin contamination of peanuts that go to market. We need immunity or near immunity to A. flavus in varieties before we can safely dispense with cultural and handling procedures that suppress the fungus. The search for higher levels of resistance or immunity to A. flavus is an important aspect of our continuing research on this problem.

QUESTIONS: Has any work been done to develop peanut varieties with a determinate fruiting habit to lessen the range of maturity among pods at digging? What are the prospects for future developments of this sort? Are any chemicals available that can be applied to peanut plants to lessen the range of maturity among pods at digging?

ANSWER: A major objective of every peanut breeding program in this country during the past 40 years has been the development of improved varieties that produced pods that were more nearly the same age at digging. Little success has been achieved thus far and I have no personal knowledge of developments that signal a major breakthrough of this sort in the near future.

There have been rumors during the past 2 or 3 years that one of our private peanut breeders has developed a productive new variety with a somewhat dwarf habit of growth that might produce pods that are more nearly the same age at digging than our standard commercial varieties. I have not seen this new peanut and consequently am not in a position to discuss it.

A number of growth regulating chemicals have been tested on peanuts during the past several years, some with the objective of preventing new pods from developing late in the growing season. Several of these

chemicals are growth retardents, and under certain conditions have been found to reduce excessive vine growth late in the season. However, I am unaware of convincing experimental evidence that these chemicals reduce the proportion of immature pods at digging and appreciably increase the market grade of the cured crop that goes to market.

We have cooperative research underway in Texas that seeks among other things to identify the chemical reactions that control peg elongation and pod development, and then to develop procedures for controlling these reactions at will under field conditions. Identification of the reactions that control peg elongation could facilitate development of practical procedures for controlling the reactions. Insofar as I am aware, our man in Texas is the only plant physiologist in the country who is devoting full time to research on peanuts.

Discussion: (J. L. Butler)

Considerable interest was expressed in the methods of measuring maturity. Although there are other methods, such as oil index, currently being used; to our knowledge none are designed for use on freshly dug peanuts. The two methods reported are for freshly dug peanuts and the results can be had within one hour. Thus, there is no likelihood of the peak maturity passing between the time the sample was taken and getting the results back. With other methods which require normal drying of the peanut before testing, this can readily happen.

The method utilizing the light transmission through a methanol extract requires using a kitchen-type blender to grind the freshly harvested peanuts in methanol, cooling to about 35-40°F, filtering and measuring the light transmission. An inexpensive (about $60.00) abridged spectrometer can be used, or the sample may be visually checked against a known standard.

The method utilizing the ratio of impedance at frequencies of 5 and 500,000 H_z requires a means of loading the freshly dug sample of peanuts to a known compression, measuring the resistance at each frequency and calculating the ratio. This method is based on the change in moisture concentration within the peanut as it approaches maturity. By utilizing these widely different frequencies, the changes in the ratio are indicative of the changes in moisture concentrations.

The Research Committee expressed desire in a continuation of the application of mold inhibitors to peanuts in the windrow. These will be intensified with adequate residue samples.

Much interest was also expressed in the "green" harvesting process. This would allow complete control of the drying process and would virtually eliminate the chance for aflatoxin contamination. Research on mechanisms for harvesting will be continued and additional experiments for drying the green harvested peanuts are planned.

STORAGE STUDIES

C. E. Holaday

In our experience we have found that the major causes of molding and aflatoxin production in peanuts is the rewetting of dried peanuts followed by poor drying conditions and storing improperly dried peanuts. Even when a load of peanuts is dried to an average moisture content of 8% or lower, if the load contains very many immature pods molding may occur since the immature pods do not dry down to the same moisture level as the more mature pods because of a much higher initial moisture. The effect of rewetting on molding is illustrated in Table 1. So the two main considerations in storing peanuts are: (1) store in a bin or house well protected from the weather particularly blowing rain and (2) make sure peanuts are properly cured with a minimum of immature pods.

The storage studies at the National Peanut Research Laboratory are cooperative efforts between the Transportation and Facilities and the Market Quality Research Divisions. The Market Quality Research Division also does special cooperative studies with the Agricultural Engineering Research Division. We have underway three studies on storage. The first of these is a cold storage test in which shelled peanuts are stored in two types of containers at four humidities and at a constant temperature of 35° F. The purpose of the study is to determine the ideal humidity and container for best quality maintenance of shelled peanuts in cold storage. Table 2 shows the treatments used in this study. Under these conditions, of course, you would not expect aflatoxin contamination nor have we found any, but we have seen "Rhizopus sp." mold on peanuts with high moisture in refrigerated storage.

As many of you are aware in the last two or three years some shellers have been rewetting their peanuts prior to shelling in order to improve milling efficiency. This practice can cause serious aflatoxin contamination unless precautions are taken. In order to acquire data on this type of treatment a study was designed to test two methods of rewetting peanuts. One was by spraying tap water on peanuts moving on a belt and the second was by aereating peanuts continuously with high humidity air. Table 3 shows the test schedule for the first rewetting method. The original moisture was 5% and at the rate of 1/2 ga. of water/min., the moisture was raised to 7 1/2 % and at 1 gal/min. rate, the moisture was raised to 10 1/2%. The flow/rate of the peanuts was 1 ton/hr. After remoistening the peanuts were stored 24 hours before shelling. There was a significant decrease in splits; but also an increase in bald-face. Our preliminary results indicate that milling quality was not improved sufficently to warrant the increased risk of aflatoxin contamination and the additional cost of the added moisture. Another point should

be mentioned and that is that this study was made during the late fall when the temperatures were 60°F and lower. This may be factor with regard to the absence of molding.

The other method of conditioning farmers stock peanuts before shelling involved aereating the peanuts with high humidity air for two weeks. Table 4 shows the test schedule for the second remoistening method. Molding occurred in those aereated with 82%, 86% and 90% relative humidity air and aflatoxin was found in the peanuts from the 90% relative humidity treatment. Other quality factors such as flavor and free fatty acids were also affected adversely with this type of treatment. Plans are to run both these tests again this year during the warmer months to determine the effect of higher temperatures.

A third study this past year was concerned with the determination of the best conditions for long term storage of farmers stock peanuts. Table 5 shows the schedule of tests. In the first 3 tests air at 60-75% relative humidity is passed through the peanuts. In the last 3 tests, which are the controls, no air is passed through. The 60-75% relative humidity range was chosen because in a previous study in which several relative humidity ranges were compared, peanuts from the 60-75% relative humidity treatment had the best quality. Aflatoxin analyses of the peanuts were made throughout the tests. In all cases the tests were negative. The preliminary results indicate that the milling quality was improved over the controls and that other quality factors such as flavor were about the same.

In conclusion, I would like to say that at the National Peanut Research Laboratory we are making every effort to carry out a research program to solve the more pressing problems of the peanut industry with regard to mycotoxins and peanut quality measurements, maintenance and enchancement. We are continuously improving our research capabilities. Just recently we have put into operation 5 environmental chambers for small lot storage studies. We welcome all ideas and suggestions from industry on how best to serve them.

TABLE 1

EFFECT OF INITIAL MOISTURE LEVEL ON AFLATOXIN CONTAMINATION IN HIGH HUMIDITY TREATMENTS

MOISTURE (% w.b.)	PERCENT SAMPLES WITH AFLATOXIN		
	SPANISH %	RUNNER %	FLORIGIANT %
> 30	3	2	1
25-30	25	1	6
20-25	31	7	6
15-20	33	9	7
< 15	29	8	5
Overall Average	24	6	4

TABLE 2

SCHEDULE OF SHELLED STOCK STORAGE TESTS 1/

Test Number	Percent Humidity	Container
ST-1	45	Bin
ST-2	45	Bin
ST-3	45	Burlap bag
ST-4	45	Burlap bag
ST-5	55	Bin
ST-6	55	Bin
ST-7	55	Burlap bag
ST-8	55	Burlap bag
ST-9	65	Bin
ST-10	65	Bin
ST-11	65	Burlap bag
ST-12	65	Burlap bag
ST-13	80	Bin
ST-14	80	Bin
ST-15	80	Burlap bag
ST-16	80	Burlap bag

1/ Duration of all tests approximately one year. Bins have low flow aeration. All tests to be sampled once each 3 months for quality measurements.

TABLE 3

FARMERS STOCK CONDITIONING STUDY

ORIGINAL MOISTURE	FINAL MOISTURE	PEANUTS FLOW RATE
%	%	
5	7 1/2	1 ton/hr
5	10 1/2	1 ton/hr

NOTES: No molding or aflatoxin
Decrease in splits
Increase in bald-face
Peanuts stored 24 hrs. before shelling

TABLE 4

EFFECT OF RELATIVE HUMIDITY ON THE MOISTURE CONTENT OF THE VIRGINIA-TYPE PEANUTS

FIRST SERIES			SECOND SERIES		
RH	MOISTURE CONTENT 1/		RH	MOISTURE CONTENT 1/	
	KERNELS	HULLS		KERNELS	HULLS
%	%	%	%	%	%
86 2/	14.8	17.6	90 2/	13.6	16.7
77	10.1	14.6	82 2/	9.7	15.7
64	6.5	11.1	69	7.6	10.8

1/ Av. of 6 samples. Initial moisture about 5%

2/ Molding

TABLE 5

SCHEDULE OF FARMERS' STOCK STORAGE TESTS

Test Number	Condition of Aerated Air
1	60-75%
2	60-75%
3	60-75%
4	None
5	None
6	None

Storage Studies

J.W. Dickens

Field studies in cooperation with the Peanut Administrative Committee and with individual shellers have shown that Aspergillus flavus growth occurs during storage of farmers' stock peanuts. Production of aflatoxin during storage of farmers' stock peanuts is causing a serious problem with aflatoxin in shelled peanuts.

One of the major causes of A. flavus growth appears to be inadequate ventilation. Many warehouses have no provision for ventilation. Condensate forms on the underside of the roof and drips on the peanuts or forms on sidewalls and wets the peanuts. Leaking roofs, improper application of insecticide sprays, collection of water in dump pits and storage of peanuts in new facilities with damp concrete floors were cited as other causes of aflatoxin production in farmers' stock peanuts during storage. Samples of molded peanuts were taken from storage which contained up to 4,000 ppb aflatoxin.

The importance of proper storage of farmers' stock peanuts was illustrated by a series of slides showing A. flavus growth on peanuts inoculated with A. flavus and stored at 20% moisture for 23, 30, 46 and 52 hours. Aflatoxin concentrations in the samples at the end of these storage periods were 0, 150, 416 and 2,177 ppb, respectively.

Possible inadequacy of fungicide sprays to prevent A. flavus growth on farmers' stock peanuts was demonstrated by a photograph which showed good control of A. flavus growth on the surface of peanuts pods dipped in a fungicide solution although there was extensive A. flavus growth inside the pods. Another slide showed excellent control of A. flavus growth on farmers' stock peanuts with storage atmospheres containing high concentrations of carbon dioxide. Other research has shown that high concentrations of carbon dioxide will also control insects. Unfortunately, most storage facilities for farmers' stock peanuts are not suitable for controlled atmosphere storage. The effects of high concentrations of carbon dioxide on peanut quality should be determined before using this method.

Bulk storage of farmers' stock peanuts may require aeration with fans to prevent moisture migration and mold growth. When warm peanuts are stored, natural convection causes warm, moist air to rise to the surface of the peanuts during cold weather. Moisture condenses on the cool peanuts near the surface and will cause mold growth during subsequent warm weather. Aeration fans may be used to draw cool air down through the peanuts to cool them and prevent moisture condensation. Estimated fixed costs for aeration systems range from $2 to

$10 per ton with operating costs of $.15 to $.40 per ton per year. Further studies are needed to determine the feasibility of aerated storage for farmers' stock peanuts.

Discussion:

Mr. C.A. McNair, Gold Kist Peanuts, asked about the feasibility of insulating the walls and roof of peanut storage buildings to prevent moisture condensation. Mr. J.W. Dickens replied that this may not work since ventilation will be necessary and ventilation probably would cool the inside of the insulation to the dew point and thus cause condensation.

Mr. Dick Johnson, CPC, asked if the shellers are aware of the problem of aflatoxin production in storage and said that there should be a Code of Good Practices for shellers.

Mr. Jim Miller, AMS, said the Peanut Administrative Committee is considering the problem and will discuss it at the next meeting.

Mr. Jim Thigpen, representing ASCS, asked about the desirability of removing LSK from edible grades. Mr. J.W. Dickens replied that it may not be economically feasible, but that removal of LSK would reduce the risk of aflatoxin contamination in shelled peanuts.

Sampling - Update

T. B. Whitaker

The mean level of aflatoxin in lots of shelled peanuts can't be determined exactly by taking samples from the lot. As a result of using samples, a certain number of good lots will be rejected as being bad (processor risk) and a certain number of bad lots will be accepted as being good (consumer or manufacturer risk). Therefore, for a given sampling plan, there is a certain probability P(M) that a lot with a given mean level, M, will be accepted or rejected. A plot of P(M) versus M (figure 1) is called an operating characteristic (O-C) curve. The O-C curve along with the prior distribution of all lot means gives the risk levels associated with a certain sampling plan.

Using model simulation, a method has been developed to determine the O-C curve for aflatoxin sampling plans. The method has been used extensively by the peanut industry over the past several years to design and evaluate aflatoxin sampling plans. Figure 2 shows O-C curves characterizing the sampling plans used by the peanut industry since 1968. Assuming lots with a mean level of aflatoxin equal to or less than 25 parts per billion (ppb) are good and lots with levels above 25 ppb are bad, the areas associated with each curve shows the progress the industry has made with the sampling program since 1968. An O-C curve representing a 48 pound single sample is shown for comparative purposes. The areas in Figure 2 representing the risk levels associated with each sampling plan are transformed into numbers of lots (Table 1) using a prior distribution of all lot means estimated from 1969 sampling data in the southeast. Table 1 shows that each year the industry has been accepting fewer bad lots (BLA) (consumers or manufacturers risk) and have let fewer lots with extremely high levels (above 55 ppb) through the system (BLA55). The table also shows that in decreasing the manufacturer's risk the industry is rejecting more total lots (TLR) and rejecting more good lots (GLR). As a result, the average amount of aflatoxin (AAA) in the lot accepted by the industry has steadly decreased.

The accuracy with which the risk levels associated with a sampling plan are predicted depends in part on the accuracy of the model to simulate the distribution of contaminated kernels and the evaluation of model parameters. One basic assumption concerning the model is that the variability in sampling results is due to sampling error alone ie: there is no subsampling and/or analytical error. But Figure 3 which is a simplified diagram of the sampling procedure, shows that there can be at least three error terms or variance components: sampling (σ_s^2), subsampling (σ_{ss}^2), and analytical (σ_a^2). The sum of the three terms gives the total variability or variance (σ_T^2) associated with sampling ($\sigma_T^2 = \sigma_s^2 + \sigma_{ss}^2 + \sigma_a^2$).

For small sample sizes (ie: 12 pounds) indications are that $\sigma_s^2 >> \sigma_{ss}^2$ or σ_a^2. However, as the sample size N increases only σ_s^2 decreases. Increasing sample size doesn't reduce σ_{ss}^2 or σ_a^2. Therefore there is a sample size N at which $\sigma_s^2 \approx \sigma_{ss}^2$ and σ_a^2. At this point it may be more productive to further reduce σ_T^2 by reducing σ_{ss}^2 and/or σ_a^2.

As a result, experiments have been conducted to quantify the three error terms σ_s^2, σ_{ss}^2, and σ_a^2. σ_s^2 was measured for multiple 12 pound samples drawn from contaminated lots, σ_{ss}^2 was measured for multiple 280 gm subsamples taken from the Dickens - Satterwhite mill, and σ_a^2 was measured making multiple assays using the standard BF procedure with a densitometer. A regression analysis indicates that σ_{ss}^2, σ_s^2, and σ_a^2 are functions of the mean level of aflatoxin. Figure 4 plots the standard deviation (σ = square root of the variance) σ_s, σ_{ss}, and σ_a versus the mean level of aflatoxin.

The coefficient of variation for sampling CV_s, for subsampling CV_{ss}, and for analysis CV_a can be determined from the variance or standard deviation in Figure 4. CV (%) = $\sigma \times 100/M$. The coefficient of variation for each step of the sampling procedure is shown in Figure 5. The CV_s reflects a 48 pound sample since that is where the industry is at the present time. In general CV decreases as the mean level of aflatoxin M increases. This is more noticeable in CV_s and CV_{ss} than CV_a. CV_a is almost constant at 22%. The CV_s and CV_{ss} may be less than an average values which represents all labs. One would expect variability among labs to be larger than the variability within a lab. Efforts are presently being made, in cooperation with the Experimental Statistics Department at N.C. State University to use 1971 sampling data to measure the variability among labs.

Discussion:

1. Dr. W.A. Pons, SMN, indicated that the CV_a was approximately 10% in his lab and that 22% shown in Figure 5 may be high. Other discussion indicated CV_a was vary greatly among labs. Results of International Check Series No. 2 indicate a CV_a = 70% for the BF procedure for 34 labs.

2. Dr. C.J. Kensler representing Arthur D. Little suggested that the CV given in Figure 5 indicates to him that it is unwise to lower the guidelines at the present time.

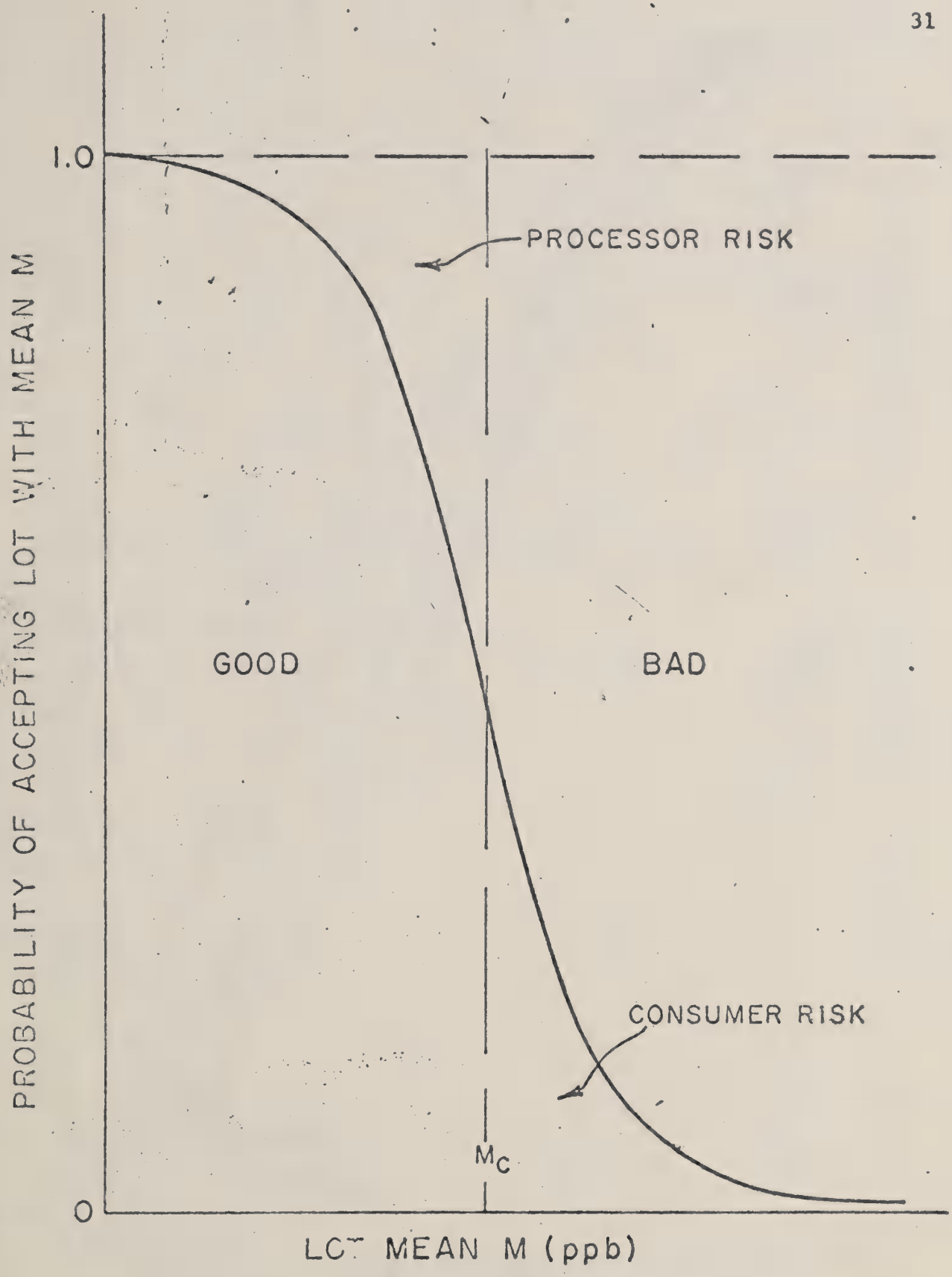


Figure 1. Generalized Operating Characteristic Curve

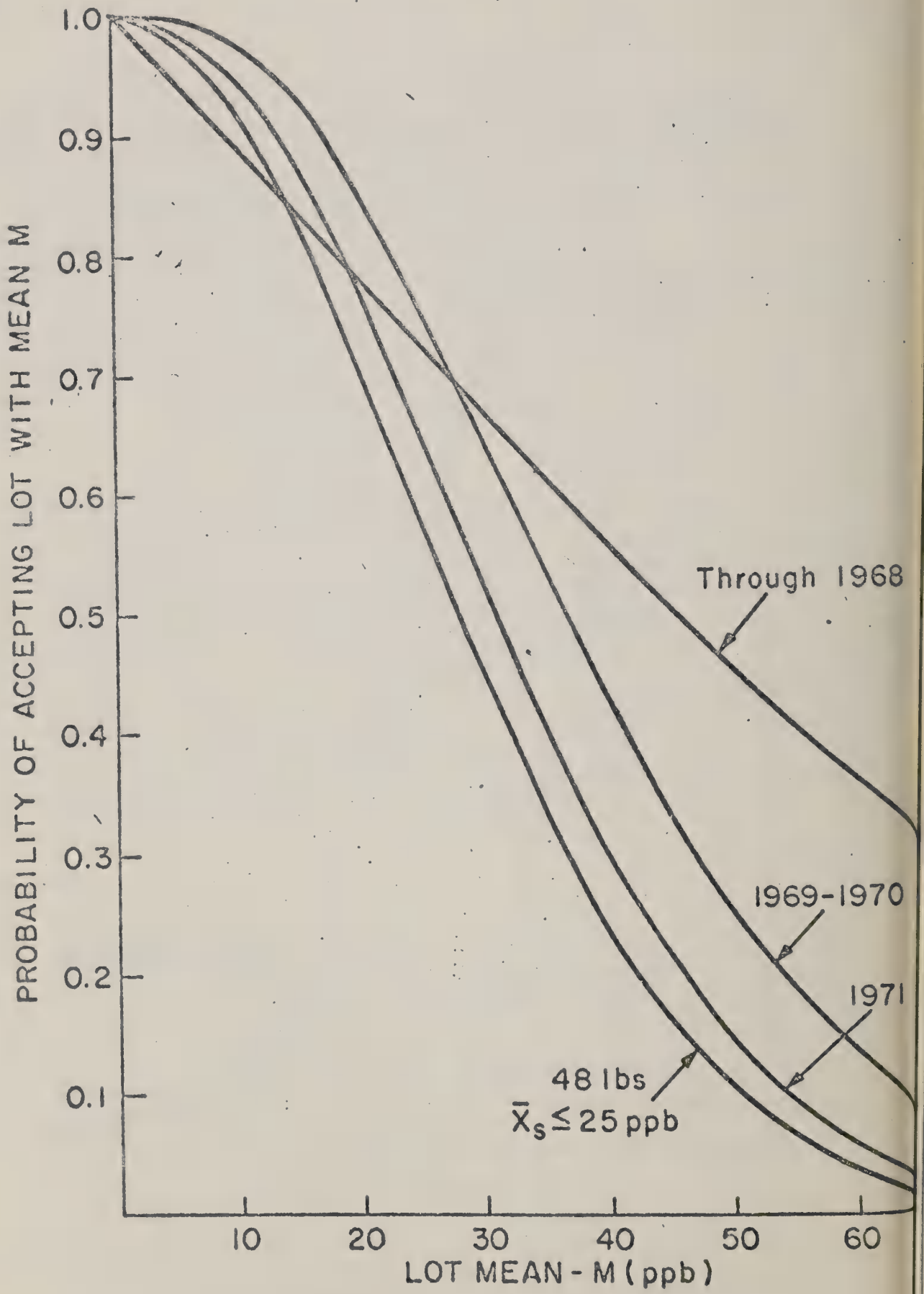

1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
PROBABILITY OF ACCEPTING LOT WITH MEAN M
Through 1968
1969-1970
1971
48 lbs
$\bar{X}_s \leq 25$ ppb
10
20
30
40
50
60
LOT MEAN - M (ppb)

Figure 2.

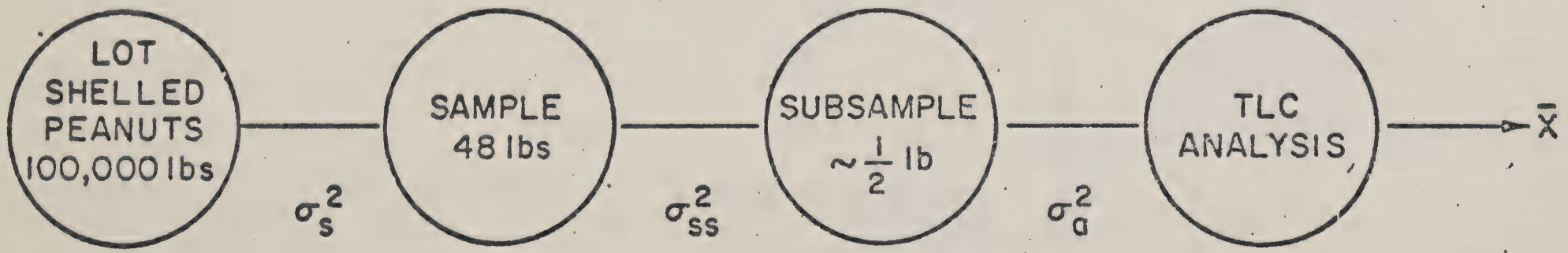
Table 1

SAMPLING PLAN COMPARISONS

	Thru 1968	1969 & 1970	1971	48 lb
TLR	778	699	855	960
GLR	288	100	169	227
BLA	441	331	244	197
BLA55	61	19	8	5
AAA	4.32	3.88	3.47	3.24

Good Lot $\leq$ 25 ppb

Bad Lot $>$ 25 ppb



$$\sigma_T^2 = \sigma_s^2 + \sigma_{ss}^2 + \sigma_a^2$$

Figure 3a. Sampling Procedure for Peanuts

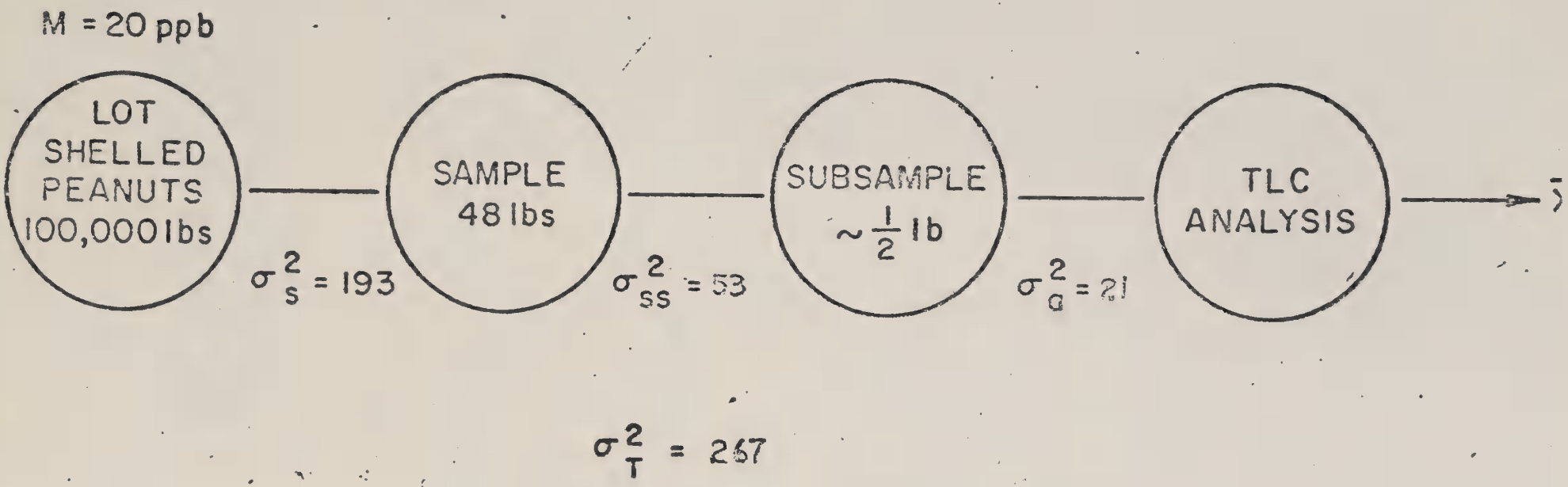
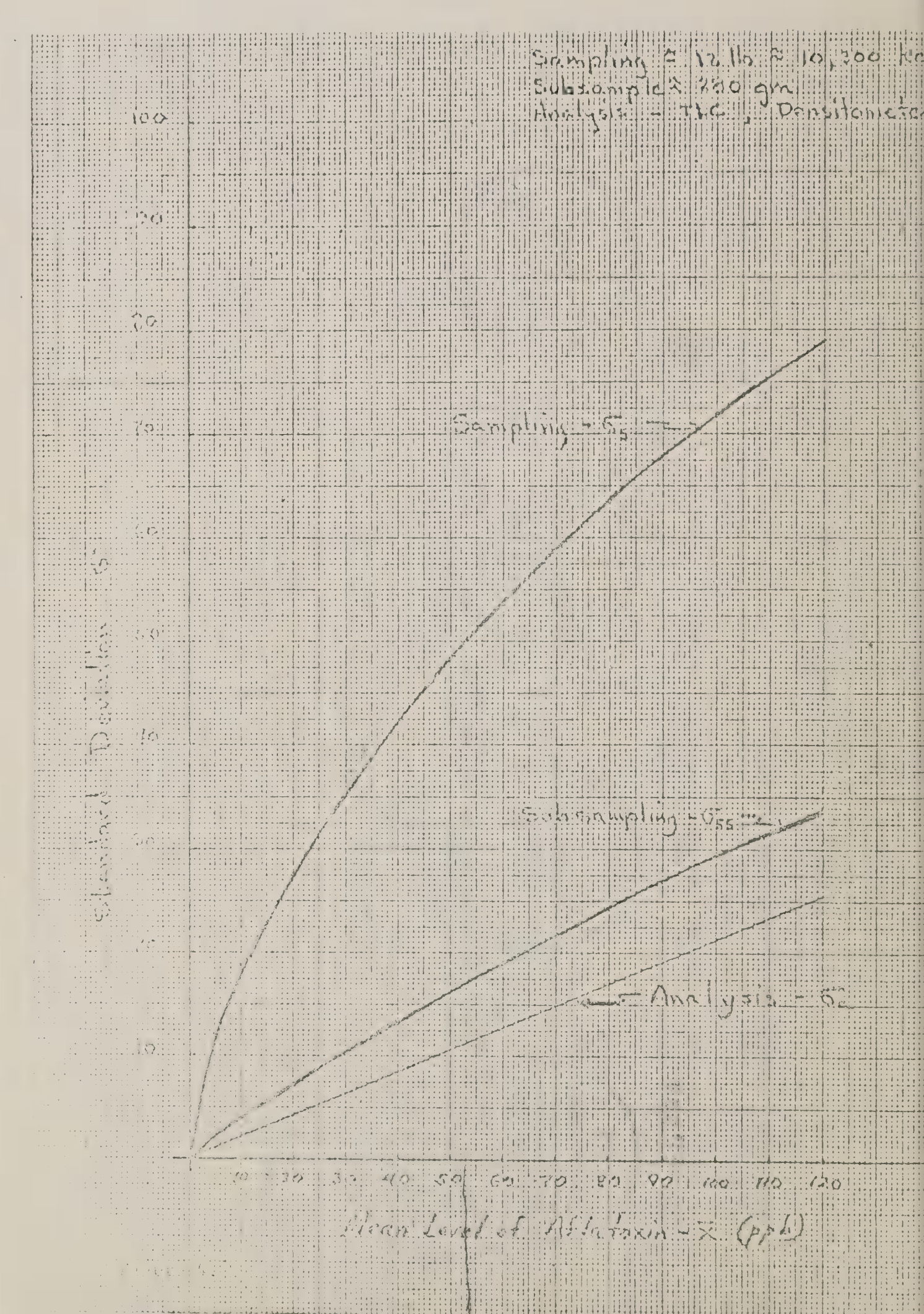

M = 20 ppb
LOT SHELLED PEANUTS 100,000 lbs
SAMPLE 48 lbs
SUBSAMPLE ~ 1/2 lb
TLC ANALYSIS
$\sigma_s^2 = 193$
$\sigma_{ss}^2 = 53$
$\sigma_a^2 = 21$
$\sigma_T^2 = 267$

Figure 3b.


Sampling ≈ 12 lb ≈ 10,200 kc
Subsample ≈ 200 gm
Analysis = TLC, Densitometr
Sampling - σ_s
Subsampling - σ_{ss}
Analysis - σ_a
Standard Deviation - σ
Mean Level of Aflatoxin - $\bar{x}$ (ppb)

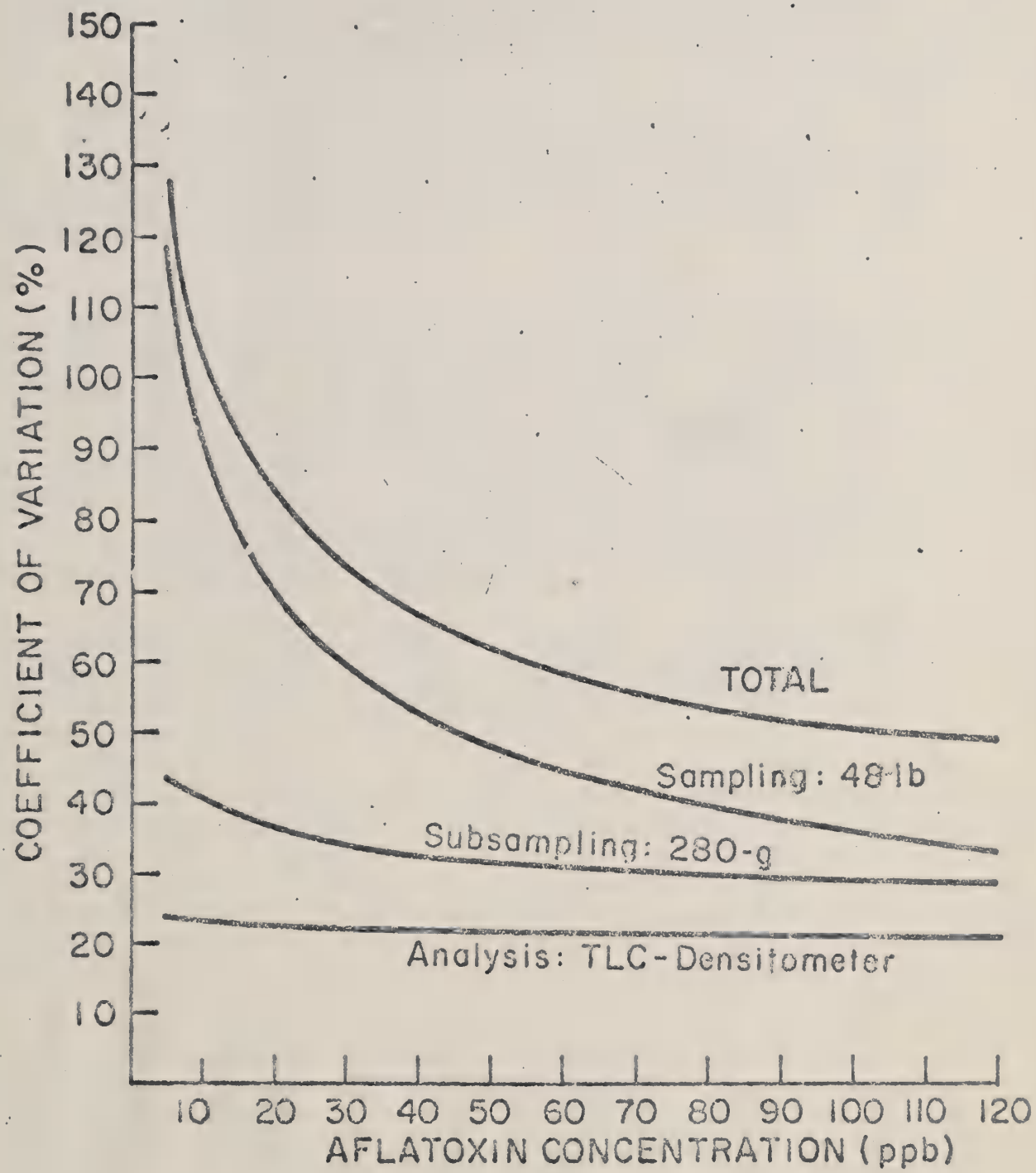

COEFFICIENT OF VARIATION (%)
150
140
130
120
110
100
90
80
70
60
50
40
30
20
10
TOTAL
Sampling: 48-lb
Subsampling: 280-g
Analysis: TLC-Densitometer
10 20 30 40 50 60 70 80 90 100 110 120
AFLATOXIN CONCENTRATION (ppb)

Figure 5.

Analytical Methodology-Millicolumn

C. E. Holaday

The peanut industry urgently needs a better screening method and possibly a better quantitative method for aflatoxin in peanuts and peanut products. We believe that the millicolumn technique offers both.

For the past 6 months we have made a major effort on improving this technique from the standpoint of sensitivity, reliability and time. As a result of our efforts we have increased the sensitivity to about 3 ppb, the reliability has been substantially improved and the time required for making a test has been reduced to 15 minutes or less.

The millicolumn used in our new procedure is essentially the same as the original millicolumn except that it is longer and is not equilibrated at 78% relative humidity prior to use. Fifty grams of sample are extracted in a Waring Blender with 100 ml. of a mixture composed of 5% acetonitrile in toluene. Ten grams of diatomaceous earth are added before extracting as a filter aid. We found that the acetonitrile-toluene mixture to be an excellent extractant for aflatoxin. Many other solvent combinations were tried, but none gave as desirable results as this combination. The extract is filtered under vacuum. It is only necessary to collect about 10-15 mls. of extract. The extraction and filtration require about 4-5 minutes. The next step is the development of the column. The lower end of the column is connected to a vacuum source and 3 ml of the extract are pipetted in at the top and pulled through. The aflatoxin will remain at the top of the column. The column is then washed with about 3 ml of hexane added at the top. The vacuum is continued until all of the solvent is evaporated from the millicolumn. The millicolumn is then removed from the vacuum source and 0.7 or 0.8 ml of a mixture containing 4% methanol in toluene is added to the top of the column. As the solvent moves down the column, it will move the aflatoxin along with it in the form of a narrow fluorescent band. When the solvent has traversed the length of the column the aflatoxin will have moved only about 10 mm from the top. The development step requires about 8-10 minutes.

In order to quantitate the aflatoxin represented on the columns, we score the column directly underneath the bands with a file. The columns are then put back on the vacuum source and washed with 3 mls of carbon tetrachloride. The vacuum is continued until all of the solvent is removed. The column is then broken and the silica gel containing the aflatoxin is dumped into a No. 541 filter paper and washed with three one ml portions of acetone which are collected in

a 50 ml flask. The acetone is removed on the steam bath or a rotovap if one is available. Five hundred ul of toluene are added to the flask to dissolve the aflatoxin. About 300 ul of the aflatoxin solution is then spotted on a thin-layer plate. Because this is 20-30 times more material than is normally spotted we developed a special technique to accomplish this. We suspend a 1 cc disposable plastic syringe with 25 gauge needle directly over the thin-layer plate (1/2 ml thick) so that the tip of the needle penetrates the silica gel. We had a metal rack made to accomplish this purpose. Another syringe is used to take up 300 ul of the solution and transfer to the suspended syringe. The spotting will then take place automatically. The plate is developed in the normal way. For standards we dilute the regular standards 100 times and spot with the syringe.

The millicolumn method works even better on peanut meal than it does on peanuts. Our results on peanut butter, however, are not as satisfactory, because of the emulsifiers, sugars, and other ingredients added to commercial peanut butter. These cause some interferences and as a result reduce the sensitivity. The method for peanut butter is essentially the same except that only 2 mls of the extract are added to the column rather than 3 as with peanuts. This makes it 1/3 less sensitive. The spots are not as clearly defined and they are slightly more difficult to quantitate than the spots from peanuts and peanut meal, however, we believe that a more effective clean-up of the aflatoxin while still on the millicolumn will overcome this.

The improved millicolumn technique, therefore offers not only a sensitive screening method but also a rapid and reasonably simple quantitative method for aflatoxin in peanuts and peanut products.

Analytical Methodology-

DEVELOPMENTS IN ANALYTICAL METHODOLOGY AT SMNR

Walter A. Pons, Jr.

My remarks today will cover some of our recent work on analytical methods for detecting and determining aflatoxins. In most instances these efforts are related to peanuts.

TOPIC I - Aflatoxins in Soapstocks

Processing of aflatoxin contaminated oilseeds removes a portion of the aflatoxins in the crude oil. Alkali refining of the oil removes these contaminants and concentrates them in the soapstock. Commercial acidulation of the alkaline soapstock to recover the fatty acids may, or may not, destroy the aflatoxins depending on the acidulation conditions. Since no method was available for determining aflatoxins in soapstocks we were asked by industry representatives to develop such a method. We did develop what we think is a reliable method and it was published in JAOCS 47, 226-230 (1970). In this work we found that in alkaline solution aflatoxin B_1 and G_1 could not be detected by ordinary aflatoxin methods since they were present as the salts of non-fluorescent hydroxy acids due to the opening of the lactone ring(s) of B_1 and G_1 in alkaline media. However, mild acidulation to pH 3 as employed in the analytical method reformed the lactone ring and resulted in essentially complete recovery of B_1 and G_1.

We applied the method to six samples of alkaline and five samples of acidulated commercial peanut soapstocks and found aflatoxin B_1 in ten of the eleven samples at levels ranging from 12 to 260 µg/kg on a moisture free basis. We found G_1 in only three of the ten contaminated samples, the G_1 ranging from 12 to 68 µg/kg.

TOPIC II - Acidulation of B_1 and G_1. (JAOCS 49, 124-128 (1972)).

The finding of B_1 in several commercial peanut soapstocks prompted us to investigate the effect of time, temperature and pH on the fate of aflatoxins B_1 and G_1 under simulated soapstock acidulation conditions. Basically we prepared solutions of pure B_1 and G_1 in dilute alkaline solution (pH 12), adjusted these solutions to pH 1, 2 or 3 with sulfuric acid and heated them at temperatures ranging from 40 to 100°C. Aliquots were withdrawn periodically from 15 to 360 minutes reaction time at given temperature and pH, and analyzed for residual B_1 and G_1 content. In agreement with observations previously reported by Dutton and Heathcote (Biochem Journal 101:21 (1966)), Ciegler and Peterson (Applied Microbiol. 1968:665), and

Pohland et al. (JAOAC 51:907 (1968)), we found that heating acidified aqueous solutions of B_1 and G_1 resulted in their conversion to the hydroxy derivatives B_{2a} and G_{2a} respectively, with the OH group in the 2-position of the terminal furan ring. We also found no M_1 to be produced by acidulation of B_1.

A kinetic study of the data showed that the conversion reaction was first order with respect to B_1 and G_1 and also first order with respect to H^+ ion concentration. The conversion reaction is strongly pH dependent, and we calculated that the time required to convert 95% of original B_1 to B_{2a} at 100°C was seven hours at pH 3, 47 minutes at pH 2 and ten minutes at pH 1. Since commercial acidulation conditions are generally in the range of pH 3-4, the need for lower pH during acidulation is indicated, i.e., if one wants to destroy or inactivate B_1. It might be mentioned that Dutton and Heathcote (Chem. and Industry 1968:418) found B_{2a} and G_{2a} to produce no detectable toxicity or liver lesions when fed to day old ducklings at 66 times the LD_{50} value of B_1 and 19 times that of G_1. We found in our work that B_{2a} and G_{2a} were quite unstable in acid solution, and in fact we were not able to detect either B_{2a} or G_{2a} in samples of commercial soapstocks which contained residual aflatoxin B_1.

TOPIC III - Rapid Detection of Aflatoxins: Gas Liquid Chromatography (GLC)

We have explored two approaches for rapid detection of either mold or aflatoxin contamination in peanuts and cottonseed. One of these is the detection of volatile metabolites elaborated by molds, using Gas Liquid Chromatography (GLC). Working with actively metabolizing Aspergillus parasiticus growing on wet (20-30% H_2O) peanuts, we swept dry, room temperature air over the sample and trapped the volatiles on an adsorbent. After heating the adsorbent and introducing an aliquot of the heated head space gas into a gas chromatograph, we found acetaldehyde, methanol, ethanol and acetone to be the principal volatile metabolites. When weight mixtures of wet moldy to dry sound peanuts ranging from 1:70 to 1:1400, were analyzed by the air sweeping and adsorbent trapping of volatiles only acetone was detected in significant amounts. Moreover, when we air-dried wet, moldy peanuts (30% H_2O) to normal moisture levels (6% H_2O), acetone and other volatile metabolites were lost, and the technique was inapplicable. Similar results were obtained with Segregation III mold contaminated dry peanuts, indicating that the technique was only applicable for detecting actively metabolizing mold.

TOPIC IV - Rapid Millicolumn Detection of Aflatoxins

In the fall of 1970 we were urged by members of the cottonseed industry to develop a rapid and simple method for detecting aflatoxins in cottonseed, hopefully within 5 minutes. We first explored C. E. Holaday's rapid millicolumn method for detecting aflatoxins in peanuts, but found it inapplicable to cottonseed due to interferences from gossypol and lipids extracted by the chloroform:methanol (97:3) extraction solvent of Holaday's method. We thought that Mr. Holaday's original idea, i.e., the use of a small column (4 mm I.D.) filled with silica gel to detect aflatoxins in a rapid chromatographic separation was a good one, and we used his basic idea in the method we developed for detecting aflatoxins in cottonseed products within 15 minutes. Basically the method involved a rapid blender extraction of the sample with aqueous acetonitrile. Acetonitrile:water is a poor lipid solvent, but an excellent solvent for extracting aflatoxins. An aliquot of the crude extract was then shaken with 3 ml of benzene and salt solution in a funnel for 30 seconds to phasically transfer the aflatoxins into benzene. This simple phasic transfer removes interferences due to the water in the solvent, separates aflatoxins from most residual interferences, and concentrates the aflatoxins 5-fold over that in the original extract. Next we dipped a millicolumn (4 mm I.D.) filled with zones of acidic alumina and dry silica gel into the benzene extract and wicked a portion onto the column. The column was then developed for 5 minutes in a chloroform:acetonitrile:2-propanol solvent and examined under long wave UV light. The presence of aflatoxins was denoted by a sharp blue fluorescent band ca 1 cm above the alumina zone. The acidic alumina served to strongly adsorb gossypol pgments in the extract, and the development solvent used gave a sharp aflatoxin band. The entire method required only 15 minutes, and the sensitivity was about 10-20% µg/kg of aflatoxin. This method was presented in October 1971 at the AOAC Meeting and will appear soon in JAOAC.

Shortly after presenting the millicolumn method just described we received numerous inquiries as to whether the technique was applicable to peanuts, corn, tree nuts and other commodities. We decided to test its applicability as a more general screening method and found that with minor modification, it was applicable to oilseeds including peanuts, peanut meal, soybeans and soybean meal, flax and sunflower; to tree nuts including almonds, pecans, walnuts, cashews, brazil nuts, filberts and pistachios; to grains including yellow and white corn and cornmeal, rice, oats, barley, wheat and sorghum.

The modification just mentioned involves a quick lead acetate treatment of the crude extract to remove interfering pigments such as chlorophyll, xanthophylls, flavones and chlorogenic acid in some agricultural products. The lead acetate treatment adds ca 5 minutes to the procedure but it gives very clean extracts and in most cases allows detection of aflatoxins at levels as low as 10 µg/kg. It is not required for peanuts but is necessary for peanut meals. Anyone interested in this rapid detection method may obtain it by writing to our Laboratory.

TOPIC V - Aflatoxins in Eggs, Tissues and Milk

Quite recently we were given an assignment to develop suitable methodology for detecting and measuring aflatoxins in eggs and tissues at levels as low as 0.1 µg/kg. The purpose of such a method was to determine in controlled feeding experiments how much aflatoxin B_1 was transmitted to eggs and tissues, and what level of aflatoxins could be safely permitted in feeds to assure no transmission into eggs or tissues. I will not attempt to describe the method but will merely state that this problem turned out to be a formidible one, particularly with eggs. The method which we eventually developed is capable of reliably measuring aflatoxins at levels as low as 0.05 µg/kg, and we have actually measured aflatoxin B_1 at a level of 0.02 µg/kg in eggs from experimental diets fed to laying hens.

We found that the method developed for aflatoxins in eggs and tissues could be adapted as a simple and fairly rapid method applicable to determining aflatoxin M_1 in milk at levels as low as 0.1 µg/L of liquid milk.

The method for eggs and tissues and milk will be submitted for publication in the near future.

Discussion:

Question (Unknown): Will SMNR millicolumn method work on peanut butter?

Answer (Pons): We have not tried it as yet for peanut butter. Dr. Pohland of FDA stated that they found it to be applicable in a few tests on peanut butter.

Question (Unknown): When will feeding experiment to check possible aflatoxin transmission into chicken eggs and tissues be done?

Answer (Dr. Senti): This is in future; planning of experiment not complete as yet.

Statement made by A. D. Campbell (FDA):

The SMNR millicolumn method is in use in Turkey and Iran to screen pistachios for aflatoxins. It has also been sent to FDA district labs as a Laboratory Information Bulletin for their use (FDA) in screening commodities.

With regard to the toxicity of B_{2a}, Dr. Campbell stated that FDA fed B_{2a} to trout and found it was not acutely toxic but had chronic toxicity. In reply to a question about toxicity of citrinin, Dr. Campbell said not much is known about Citrinin toxicity as yet, but it is a carcinogen.

Question (Kensler): What is sensitivity of SMNR methods for aflatoxins in eggs and milk?

Answer (Pons): Eggs down to 0.05 ppb; milk is 0.1 ppb without column chromatographic cleanup and lower if column cleanup is used.

Analytical Methodology - Comments from AMS

AMS/PAC Peanut Aflatoxin Analytical Program

John B. Wegener

We have 10 laboratories analyzing peanuts, peanut products and other commodities for aflatoxin.

We are currently testing all of the Brazil nuts imported into this country, some 300 lots per year. This is the fifth year for this program and we feel it has been very sucessful. No lots have been found the last two years containing actionable levels of afltoxin. In the earlier years we rejected some shipments.

We are about to start on a similar program on pistachios and interest has also been shown by the industry to have pecans tested.

Our 10 AMS laboratories run about 30,000 samples of peanuts, 2,000 samples of peanut butter and 1,000 samples of other odds and ends each year for aflatoxin.

The USDA purchases of peanut butter, for the last two years, had no measurable levels of aflatoxin, and we consider this to be a mark of the success of the aflatoxin program.

Our original lot sample size has increased over the years from 2 lbs. 5 lbs., 10 lbs., 12 lbs., 24 lbs., to the present 48 lbs. projected by Mr. Fred Whittaker.

Initially our analytical method was rather crude - we had pure standards for only aflatoxin B_1 and G_1. We didn't know that B_2 and G_2 existed. The sensitivity was approximately 10 ppb. Today we have pure standards for the four aflatoxins and have increased the sensitivity to 2 - 3 ppb on our TLC plates. So we have made some progress. Of course, Walter Pon's method on eggs, sensitive to 20 - 50 ppt, makes our work look rather crude again.

Under the sponorship of PAC we exercise technical control for them over all laboratories who qualify as a PAC laboratory. There are approximately 50 laboratories in this group - 10 AMS, a half dozen commercial chemists, and the balance manufacturers or user's labs.

There are about 3,000 lots of peanuts produced each year by the shellers. I estimate that the above laboratories run well over 100,000 analyses on these peanuts each year. That represents a lot of work and a big expenditure of money.

In order to get a picture of the analytical capability of these laboratories, we have had an active quality assurance program for the past four year. The analytical method used is the BF, and I have personally visited all of these laboratories to observe their technique and equipment. We send out collaborative samples periodically. The samples are deoiled peanut meal or flour, finely enough ground to pass a 100 mesh screen. We chose this form of sample rather than a truly homogenous one such as peanut butter because over three-fourths of these laboratories do not routinely run peanut butter.

The coefficient of variance for these 50 laboratories has ranged from 25-40%. The C. V. for our 10 AMS labs, for the same samples, has ranged from 20-30%. The best agreement was reached when a prediluted aflatoxin standard was supplied with each sample. A C. V. of 25% was obtained. Because of the difficulty in providing standards we have discontinued this and as a result our C. V.'s have increased again.

We have observed that as the average level of aflatoxin in the collaborative test sample decreases the C. V. tends to increase. This is due, I believe primarily to the sensitivity of the method rather than the competency of the laboratories.

SAMPLE	AVERAGE	S. D.	C. V.
W-1-70	36.6	± 9.3	25.4
W-2-71	22.3	± 9.3	42.1

In other words, applying 1 S. D. to the average of sample W-1-70, 36.6 ± 9.3 gives a variation of 25.3 to 45.9 and treating sample W-2-71 in the same manner results in a much greater spread.

We believe the PAC aflatoxin program has been quite successful, and the industry should be commended on their efforts. We believe the PAC laboratories have done good analytical work, in spite of C. V.'s of 30 to 40, and they too should be commended on this showing.

Analytical Methodology - Comments from FDA

A. D. Campbell

Adequate methodology is currently available for the analysis of aflatoxins to carry out FDA's mycotoxin regulatory programs. For this reason we do not have continuing research projects on aflatoxin analytical methods but work on specific problems as they arise. In this regard, many of you are aware of the apparent discrepancy between the CB and the BF procedures for peanuts and peanut products as indicated by the results from the International Aflatoxin Check Analysis Series. In several of the samples, the CB procedure gave higher results than the BF. In these instances the levels of contamination were high enough so that the differences would not be of practical significance for regulatory decisions. Preliminary results from the follow up peanut butter sample which had a level of contamination very close to 20 ppb indicate no significant difference between the two methods at this level of contamination. We are planning some research employing radio actively tagged aflatoxin which we feel will provide adequate data so that a practical answer can be obtained to this problem.

We direct most of our analytical research efforts toward mycotoxins other than aflatoxins and toward problems where methodology with the necessary sensitivities are not available, such as methods for the detection of aflatoxin M in milk and milk products and aflatoxin in edible tissues of meat animals.

We are enthusiastic over the potential use of the rapid screening aflatoxin method ("millicolumn" method) developed for cottonseed by the USDA Southern Regional Research Laboratory. I had just returned from a special assignment in Turkey and Iran involving aflatoxin contamination of pistachio nuts when I listened to Walter Pons' presentation of the method at the AOAC meeting. Walter visited our laboratories after the meeting and as a result of our discussions and the need for a rapid procedure for pistachio nuts, Walter and Stan Nesheim adapted the method to pistachios by the incorporation of a lead acetate cleanup step. This modified version was immediately supplied to interested parties in Turkey and Iran. A little work in our laboratories indicated its desirability for more general application and we are taking steps to introduce it as a first step in the analysis of various samples in our regulatory program. It is our current feeling that it will find an effective use in these programs. (Copies of the method as we employ it can be obtained upon request.)

We are very interested in the current work being carried out by the USDA and by industry in the investigations on the detoxification of cottonseed, cottonseed meal, and coconut meal. The results from ammoniation studies appear promising at this time. Food Additive Regulations already exist for ammoniated cottonseed meal and ammoniated rice hulls so that the process is acceptable for some animal feed treatments. Our concern is with the potential toxicity of the aflatoxin alteration products resulting from the treatment. As you have already learned here today two year rat studies being conducted by the United States Department of Agriculture are nearing completion. Industry has conducted experiments with dairy cattle and a joint industry-USDA-FDA-University project is investigating the effect of feeding edible tissues (liver and red meat) from these cattle to rainbow trout.

Dr. Cole's report of finding citrinin as a contaminant of peanuts is of considerable interest. Professor Krogh of the Royal Veterinary and Agricultural University in Copenhagen V. Denmark is about ready to conduct a collaborative study on a method for this mycotoxin under the auspices of IUPAC. Some of you may be interested in collaborating in this study.

Removal, Inactivation, and Protective Factors

H. L. E. Vix

Protective Factors

This area of research centers on biological investigations to determine whether there are protective factors in peanuts which reduce the carcinogenicity or toxicity of aflatoxin. To this end, on June 24, 1971, ARS awarded a 2 and one-half-year contract ($65,916) to the Wisconsin Alumni Research Foundation, Madison, Wisconsin (Project Leader Dr. Paul C. Rees). The objective of this contract is to determine if peanuts and peanut products contain factors that protect rats from adverse physiological effects, including hepatomas, caused by long-term ingestion of aflatoxin.

The protocol for the long-term study as presently defined will utilize the Charles River Inbred Strain CDF (Fisher 344 derived) male rats; aflatoxin feeding levels of 0, 5, 15, 50, and 200 ppb; and peanut butter supplementation at 25% of total diet.

Diets include the following:

a. Semi-synthetic basal with simulated commercial peanut butter at the four levels of aflatoxin contamination from peanut sources.

b. Semi-synthetic basal at zero level (aflatoxin) and the four levels of aflatoxin contamination obtained by addition of pure aflatoxin to correspond to the composition and levels of aflatoxin in (a) above.

c. Semi-synthetic basal with simulated commercial peanut butter at zero level (aflatoxin) and the four levels of aflatoxin contamination obtained by addition of pure aflatoxin to correspond to the composition and levels of aflatoxin in (a) above.

d. Semi-synthetic basal with cottonseed meal at zero level (aflatoxin) and the four levels of aflatoxin contamination obtained from a cottonseed source.

The basal ration and peanut butter or cottonseed meal supplement are all formulated to provide 20% protein.

Observations on the animals include body weights, food consumption, hemotology, and other biological tests such as enzyme studies, acute organ damage, capillary fragility, urinalysis and gross and histological examination of tissues. All necessary materials, such as peanut butter, cottonseed meals, and pure aflatoxin have been obtained.

Preliminary feeding tests were started December 23, 1971, and after eight weeks, body weight and food consumption data with diets a, b, and c, showed no abnormal differences as a result of feeding aflatoxin at the test levels chosen. Also no chemical effects of aflatoxin feeding have been observed.

A definite body weight, food consumption and feed efficiency effects were noted in the animals fed cottonseed meal (diet d). This was apparently due to palatability problems resulting in low feed consumption and possibly to the high free-gossypol content of the control cottonseed meal. The control cottonseed meal was apparently the causative agent and an attempt to replace this meal with a more suitable material has not been successful. However, continued efforts are being made to obtain a more suitable cottonseed meal. The first 8 to 10 week results are to be considered as preliminary, and more Fisher 344 derived rats have been ordered to initiate the long-term feeding tests.

Removal - Physical Separation

It has been our experience, and that of others who have reported, that the vast majority of the aflatoxin in contaminated peanuts (and also in cottonseed) generally resides in a relatively small number of seeds. This should afford an exceptional opportunity for effectively, yet economically, reducing aflatoxin content by mechanical removal of those few seeds or kernels that may have become contaminated. It has been demonstrated repeatedly that the levels of aflatoxin in peanuts correlate with the number of shrivels, rancid and discolored kernels. When these substandard materials are separated from the rest of the nuts, the remaining high quality nuts are virtually low or free of aflatoxin. This culling of substandard peanuts is typically accomplished at shelling plants, by removing discolored and shrivelled kernels by hand-sorting or by electronic-sorting devices which examine each kernel individually. Segregation after blanching or after splitting peanut kernels through electronic-sorting, as practiced in the U.S. peanut industry today, does offer a means of removing the

small percentage of aflatoxin contaminated peanuts and renders the rest of the processed peanuts (over 95%) as a product with an acceptable level of aflatoxin; i.e., below guideline tolerances. But the blanching procedures (without roasting) used have a somewhat adverse effect on the ultimate flavor of the peanuts, especially when such peanuts are processed into peanut butter. As a result of this problem ARS initiated a research contract ($111,667) on June 1, 1971, at the Agricultural Experiment Station, Oklahoma State University, Stillwater, Oklahoma. (Project Leader - Dr. B. L.Clary, Dr. G. H. Brusewitz, and Dr. G. O'Dell associates.) The objective of this research is to develop a pilot plant scale blanching process (without roasting) in which aflatoxin contaminated kernels can be removed by electronic sorters to obtain blanched peanut kernels with flavors that are acceptable for food products. The contract is for two and one-half years and embodies research to evaluate four types of treatments for the removal of skins from kernels. These four treatments are heating, wetting, chemical treatment (such as mild alkalies), and freezing (cryogenic). For each treatment different levels of moisture content, temperature and holding times are being investigated.

The first six months of this contract have been devoted to the design and development of equipment for conducting the various treatments. Lots of selected peanuts with and withoutaflatoxin have been obtained. Research on all four treatments are underway, and tests on the best heat treatments for blanching procedures are nearly completed. The successful blanching procedure developed for the different treatments will be tested for removal of aflatoxin contaminated peanuts. An ESM (Electronic Sorting Machine) color sorter has been obtained and calibrated for use in this research.

One phase of electronic-sorting being investigated is the type of light to be used. Dr. Brusewitz has recently investigated, through spectrophotometric research, effects of visible, fluorescent (ultraviolet) and infrared light on high aflatoxin contaminated peanuts with and without skins. Evaluation of these four types of light is not yet completed.

As the research progresses, lots of sorted blanched peanuts with acceptable levels of aflatoxin will be tested for moisture, lipids, oil, peroxide value, and free fatty acids. Organoleptic tests will be determined on peanut butter produced from those lots of peanuts with less than 20 ppb of aflatoxin.

After evaluation of all results from the four treatments, a suitable method will be selected and developed on a pilot plant scale for continuously blanching peanut kernels and removing aflatoxin contaminated kernels to produce materials suitable for food uses such as peanut butter.

We are working closely and vigorously with the contract leaders. Dr. Clary and Dr. Brusewitz are scheduled to visit us on May 11, 1972 to review and discuss their progress to date.

Another physical separation technique which is showing some promise for separating aflatoxin contaminated material from cottonseed is a Zig-Zag Separator. We now have a pilot plant installation at our Laboratory. We have obtained a pure meat (whole and cracked) fraction, free of hulls, representing about 55-60% of the available meats, with a level aflatoxin below 10 ppb from a lot of cracked, hulled cottonseed with a total aflatoxin content of 300-400 ppb. Later the unit will be tested for possible removal of aflatoxin contaminated peanut kernels from contaminated lots. Some previous work with peanuts using devices similar to the Zig-Zag Separator has been reported.

Still another physical separation technique under study at our Laboratory is a liquid-flotation process. Limited success has already been achieved in concentrating the aflatoxin contaminated, delinted cottonseed in a fraction (15% by weight of the original material) which floats on the surface of the liquid medium.

Removal - Solvent Extraction

Good potential for removing aflatoxin is offered by extraction with solvents during processing of various oilseeds such as peanuts to oil and meal. Current processing practices of contaminated peanuts and cottonseed, either mechanical expressing or extraction with commercial hexane, leaves in the defatted meal the vast majority of aflatoxin that may be present in the seed.

Aflatoxins are not soluble in hydrocarbon solvents such as hexane which are usually used in processing oilseeds, but they are readily soluble in many polar solvents.

Four solvent extraction processes have been studied at our Laboratory, and results have been published for removal of aflatoxin from contaminated seed such as peanuts. They are:

1. A solvent system of acetone, hexane, and water (such as 54-44-2% by weight, respectively) was found (Gardner, Koltun and Vix)

to remove aflatoxin readily and quantitatively from prepressed peanut meal. Levels about 25 ppb were achieved for contaminated prepressed peanut meal (207 ppb).

2. A 70% acetone-30% water (by weight) mixture (Pons and Eaves) extracts gossypol and aflatoxin from aflatoxin contaminated flaked cottonseed meats. Reductions of aflatoxin by 96-98% have been obtained. The essentially full-fat product, now practically free of any aflatoxins that may have been present, can be processed for oil removal by conventional means. This solvent system is potentially applicable to peanuts and other oilseeds as well.

3. A binary solvent system of 90% acetone-10% water (by weight) has been used (Gardner, Koltun, and Vix) to effectively remove aflatoxin to a level below 10 ppb in aflatoxin contaminated peanut meal (113 ppb originally).

4. A binary solvent system of either 80% isopropanol and 20% water, or the azeotrope 87.7% isopropanol and 12.3% water (by weight) has been studied (Dollear and Rayner) for the removal of aflatoxin from contaminated peanut meals. The 80-20 mixture more easily removed the aflatoxin to acceptable levels; in some cases as low as 3 ppb.

Of the four approaches we feel that the aqueous isopropanol extraction of peanut meal is the most promising for several reasons:

A. The oil product is not involved.

B. Isopropanol has been approved by FDA for producing an edible fish protein concentrate.

C. Levels up to 250 ppm of isopropanol are allowed in the fish protein concentrate. Thus drastic desolventization is not required.

D. Little odor or undesirable flavor remains in the isopropanol extracted meal.

E. It is believed that the meal quality from nutritional standpoint is not generally affected.

F. Some conventional extraction equipment with modification can be used.

The probably cost of an installation for extraction of 100 tons of meal per 24-hour day ranges from $500,000 to $700,000. Operating costs (including amortization) for an operation of 250 days per year is estimated at about $13.00 per ton of meal extracted with isopropanol.

At our Laboratory material extracted from the aflatoxin contaminated cottonseed meal with an 80% isopropanol-20% water solvent system was treated with various reagents to inactivate the aflatoxin present. The intended purpose of this inactivation is to eliminate a serious disposal problem and to minimize weight loss of extracted meal. Several treatments have been found to be effective for destruction of aflatoxin in the aqueous isopropanol extraction. Probably the most promising is that with calcium hydroxide. For example, treatment with 3% calcium hydroxide at 100°C for one hour destroyed essentially all the aflatoxin in the diluted extract.

As an outgrowth of Dr. Goldblatt's presentation entitled "Progress in Elimination of Aflatoxin from Peanut Products" at the meeting of the African Groundnut Council, Dakar, Senegal, March 24, 1971, a delegation representing European and West African oilseed interests visited our Laboratory in New Orleans in February 1972. They were deeply interested in our extraction work to remove aflatoxin and found our research results and suggestions to be of great benefit to them. As an outgrowth, they are looking into the potential use of a ternary azeotrope consisting of hexane-isopropanol-water in the proportions of 84:12:4 (by weight) for the removal of aflatoxin in contaminated peanuts. They are also looking into the addition of aqueous ammonia to the ternary azeotrope to effect a still lower level of aflatoxin (as low as 25 ppb) in the extracted meal. This system was of particular interest to DeSmet because this European firm has a patented refinery process using this ternary azeotrope.

Noteworthy of mentioning is that at our Laboratory we have successfully prepared, by classification techniques, a white, bland protein (68-70% protein) from unblanched, finely ground, direct hexane extracted flaked peanut kernels. Both liquid and air classification are being investigated.

Besides achieving a protein concentrate fraction free of skins, we are looking into the distribution of aflatoxin in the "overs" (protein concentrate) and "unders" (fractions). We believe for several reasons (some evidence already obtained) that we might direct most of the aflatoxin in contaminated peanut meal to the "unders," particularly through liquid classification using only hexane.

Inactivation

Significant reduction in aflatoxin has been obtained in contaminated peanut kernels under roasting conditions which simulate those that might be used for producing peanut butter.

Our Laboratory has two publications reporting the results of such research: one authored by Lee, Cucullu, and Goldblatt; and the other by Lee, Cucullu, Franz, and Pons.

Published results of additional research (Mann, Codifer, Gardner, Koltun, and Dollear) at our Laboratory show the rate of aflatoxin destruction in contaminated cottonseed meal by a treatment at 100°C for a range of moisture from 6 to 30% and a treatment time up to two and one-half hours. The most severe treatment reduces the aflatoxin level from 144 ppb of B_1 to about 20 ppb. Such treatments have a similar effect on the reduction of aflatoxin in contaminated peanut meal.

However, it was concluded that the application of heat, moisture, and time alone does not provide a satisfactory method of inactivating aflatoxin in oilseed meals. The addition of appropriate chemicals is necessary. To this end, many chemical treatments were evaluated for the inactivation of aflatoxin in contaminated peanut and cottonseed meals. Results revealed that certain chemical treatments, particularly alkali treatments, showed promise for achieving inactivation of aflatoxin.

Of all the chemical treatments studied, ammoniation showed the best potential and, hence, extensive work was carried out in the Laboratory pilot plant and in cooperation with industry. This work has been summarized in a publication entitled "Inactivation of Aflatoxin in Peanut Meal and Cottonseed Meal by Ammoniation" by Gardner, Koltun, Dollear, and Rayner, and is also covered in U.S. Patent 3,429,709.

Aflatoxin (111 ppb) in peanut meal can be inactivated in the range of undetectable to <5 ppb under rather mild conditions. These conditions are: time, 15 minutes; moisture, 15%; temperature, 160°F; and ammonia pressure, 42 psig. The nitrogen content of the meal was increased by about 0.5% as a result of the treatment.

For some pressing reasons, contaminated cottonseed meal was used as a model meal. Tons of contaminated cottonseed meal (519 ppb) have been successfully inactivated to levels from none detected to 3-6 ppb by treatment with ammonia. The optimum conditions to achieve

the desired reduction were: treatment time, 30 minutes; moisture, 12-14%; temperature, 210-250°F; and ammonia pressure, 30 psig. The nitrogen content of the meal was increased by 0.8-1.0% as a result of ammoniation.

The probable cost of an installation for ammoniation of 100 tons of contaminated meal per 24-hour day is estimated to be about $200,000.

Operation costs (including amortization, etc.) for an operation of 250 days per year is estimated to be about $7.00 per ton of meal ammoniated.

With the assistance of our Western Laboratory (WMN), FDA protocol was established and necessary feeding tests with the ammoniated cottonseed meal are underway. These are:

Rat. Two years; conducted at WMNRD. Commenced June 1970. Results of 18 months of feeding rats indicate no detrimental effect from the ammonia treatment.

Trout. A one-year trout feeding trial was begun in May 1971 by Professor R. O. Sinnhuber at Oregon State University. In this test cottonseed meal was used. When examined after 8 months, the trout receiving the aflatoxin contaminated cottonseed meal in their ration had essentially a 100% incidence of hepatomas. The trout receiving treated aflatoxin contaminated cottonseed meal showed a low incidence of hepatomas, 1/20 to 3/20. Three treatments were included in the test: (1) ammoniation, and extraction with (2) aqueous isopropanol and (3) extraction with aqueous acetone. However, there was also a low incidence of hepatomas (1/20) from the control ration which presumably contained no aflatoxins. Accordingly, Professor Sinnhuber suspects cross-contamination and he is repeating the test.

Discussion:

Question (Kensler): What were aflatoxin levels in the feeding tests with peanut butter (WARF Contract)?

Answer (Vix): 0, 5, 15, 50, and 200 ppb.

Question (Kensler): Will wet blanching tend to induce bacterial contamination in peanuts?

Answer (Vix): Yes; but the drying condition used in wet blanching can be a means of control.

Question (Unknown): Is any inactivation treatment other than calcium hydroxide being used on Copra?

Answer: No one had any information. Dr. A. D. Campbell stated that FDA examined calcium hydroxide treated Copra via chick embryo test and found no toxicity. Treated Copra had 0 PER.

With regard to ammoniation of cottonseed meal, Dr. A. D. Campbell (FDA) stated that they found the ammoniated product to contain no measurable aflatoxins, and it was non-toxic in chick embryo test. He thinks the ammoniation approach is quite promising.

Comments from Research Committee of National Peanut Council

G. F. Hartnett

Mr. Hartnett indicated that the Research Committee is particularly interested in the following subject areas:

1. Genetic resistance and natural inhibitors
2. Harvest, drying and curing
3. Storage
4. Blanching
5. Loose-shelled kernels

The Committee will file a statement on their reaction to these progress reports and their recommendations for future work. (Secretary's Note)

PARTICIPANTS

Federal

USDA ARS W. K. Bailey, Beltsville, Md.
J. L. Butler, Tifton, Ga.
R. J. Cole, Dawson, Ga.
J. W. Dickens, Raleigh, N. C.
L. Feinstein, Hyattsville, Md.
C. Golumbic, Hyattsville, Md.
C. Holaday, Dawson, Ga.
W. A. Pons, Jr., New Orleans, La.
H. W. Schroeder, College Station, Texas
F. R. Senti, Washington, D. C.
H. L. E. Vix, New Orleans, La.
J. M. Wells, Beltsville, Md.
T. B. Whitaker, Raleigh, N. C.

AMS W. J. Higgins, Washington, D. C.
E. M. Knapp, Washington, D. C.
J. S. Miller, Washington, D. C.
J. B. Wegener, Washington, D. C.

HEW FDA A. D. Campbell, Washington, D. C.
A. E. Pohland, Washington, D. C.

Industry

R. Allen, E. J. Brach & Sons, Chicago, Ill.
J. Currier, National Peanut Council, McLean, Va.
G. P. Donaldson, Georgia Peanut Commission, Tifton, Ga.
G. B. Freeman, Southwestern Peanut Shellers, Dublin, Texas
G. F. Hartnett, G. F. Hartnett & Co., Inc. & NPC, Northfield, Ill.
M. S. Hardwood, M&M/Mars, Albany, Ga.
A. F. Hidalso, Proctor & Gamble, Cincinnati, Ohio
J. E. Hunter, Proctor & Gamble, Cincinnati, Ohio
G. E. Jenkins, Tom's Foods, Ltd., Columbus, Ga.
G. R. Johnson, CPC International, Inc., Englewood Cliffs, N. J.
C. J. Kensler, A. D. Little, Inc., Cambridge, Mass.
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